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Immunization of Calves Against Enterotoxigenic Colibacillosis by Vaccinating Dams with Purified K99 Antigen and Whole Cell Bacterins†

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Pregnant cattle were either vaccinated subcutaneously with (i) a suspension of purified *Escherichia coli* K99 pili, (ii) a Formalin-killed whole cell bacterin containing enterotoxigenic *E. coli* strain B44 (O9:K30;K99:H-), or (iii) a bacterin containing six different strains of bovine enterotoxigenic *E. coli* (multiple-strain bacterin), or were left as nonvaccinated controls. After birth, calves were allowed to nurse their dams and, at 12 to 14 h of age, were challenged orally with 10^{11} cells of enterotoxigenic *E. coli* strain B44. Colostral antibody titers were determined against K99, K30, and O9 antigens of B44. In the nonvaccinated control group, 9 of 10 calves developed diarrhea and died within 24 to 72 h. Similarly, all six calves in the multiple-strain bacterin group developed diarrhea and four died. In contrast to calves in the two groups mentioned above, calves nursing cows vaccinated with either purified K99 or the homologous whole cell bacterin were protected against fatal diarrhea. There was a highly significant correlation ($P < 0.0005$) between protection against fatal diarrhea and K99, but not K30 or O9 colostral antibody titers. Vaccination of cows with either purified pili or whole cell preparations containing sufficient K99 antigen may provide a means of preventing enterotoxigenic colibacillosis in calves.

It is generally accepted that colonization of the mucosal surface of the small intestine by enterotoxigenic *Escherichia coli* (ETEC) occurs without tissue invasion and is a necessary early stage in the pathogenesis of diarrhea (14). Colonization appears to be dependent upon the ability of the involved strains to adhere to the villus epithelium. Attachment to the epithelial cell surface is mediated by pili known as: the K99 antigen on bovine, ovine, and some porcine ETEC (2, 15, 16, 19, 24, 26); K88 and 987P antigens on porcine ETEC (12, 13, 21); and fimbriae (3), referred to as colonization factor antigens I and II, on human ETEC (5-7). These pili and fimbriae, which are quite distinct from the classical polysaccharide capsular K antigens, are composed primarily of protein and are serologically unrelated to each other (2a, 10, 27).

Several earlier studies showed that immunization of pregnant cows with whole cell bacterins containing ETEC prevented fatal diarrhea in nursing calves (18, 20). However, the antigenic component(s) of the bacterial cell which stimulates the formation of protective colostral antibody has not been clearly defined. Myers found a correlation between protection against diarrhea in calves and the level of passive antibody against the polysaccharide K30, but not the K99 or O9 antigens, of ETEC strain B44 (O9:K30; K99:H-). In contrast, lacteal immunity against K99, K88, and 987P pili prevented severe diarrhea and death in suckling piglets experimentally challenged with ETEC possessing the homologous colonization factor (12a, 17, 22, 25). The objectives of this study are, first, to report that lacteal immunity to K99 prevents diarrhea and death in calves experimentally challenged with ETEC strain B44, and second, to compare the levels of protective immunity induced by: (i) purified K99, (ii) a Formalin-killed bacterin containing the organism used for challenge, and (iii) a Formalin-killed whole cell bacterin containing six different strains of bovine ETEC.

MATERIALS AND METHODS

Cows. A herd of 35 pregnant Hereford and Angus cows was assembled for the experiment. Ten cows were left as nonvaccinated controls. All other cows

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were inoculated subcutaneously above the left shoulder twice with the experimental vaccines, approximately 3 and 6 weeks before the onset of calving in the herd.

Preparation of experimental vaccines. The following three experimental vaccines were used.

(i) Purified K99 was prepared from *E. coli* strain 1474 (K-12 K99$^+$) grown in a 12-liter fermentor (New Brunswick Scientific, New Brunswick, N.J.) by the method of Isaacson (11). Each dose, which consisted of 10 mg of K99 suspended in 10 ml of saline-Formalin (0.05%) solution, was administered without adjuvant.

(ii) In the preparation of Formalin-killed homologous whole cell bacterin (HWCB), *E. coli* strain B44 (O9:K30; K99:H-), which produces heat-stable but not heat-labile enterotoxin, was used. One liter of a 24-h Trypticase soy broth (Baltimore Biological Laboratory [BBL], Cockeysville, Md.) culture of strain B44 which contained 1×10^9 viable cells per ml was centrifuged, and the pellet was suspended in 50 ml of phosphate-buffered saline (PBS), pH 7.2, containing 0.4% formaldehyde. After 24 h, the cell suspension was checked for sterility and then precipitated with an equal volume of 10% $AlK(SO_4) \cdot 12H_2O$. Each cow was vaccinated with 10 ml of the final suspension, which contained the equivalent of 1×10^{11} bacterial cells.

(iii) For the multiple-strain bacterin (MSB), a Formalin (0.3%)-killed whole cell bacterin which contained equal amounts of six K99$^+$ ETEC strains of the serogroups O8:K25, O8:K85, O9:K35, O20:K?, O101:K28, and O101:K30 was supplied by Fort Dodge Laboratories, Fort Dodge, Iowa. The concentration of bacterial cells was not disclosed but was thought to be equivalent to 12 mg of dried cells per ml (L. L. Myers, Proceedings of the Second International Symposium on Neonatal Diarrhea, in press). Each dose consisted of 5 ml of bacterin.

Challenge inoculation. Studies carried out by others on the efficacy of *E. coli* vaccines were hampered by the inability to consistently reproduce severe enterotoxigenic colibacillosis in colostrum-fed calves (8, 19). Therefore, preliminary experiments were done by using several different strains of bovine ETEC cultured on different media to challenge calves at various ages. By using the following method, we were able to consistently reproduce fatal enterotoxigenic colibacillosis in colostrum-fed calves, which was characterized by the presence of large numbers of ETEC attached to the intestinal mucosa (S. D. Acres and J. E. C. Bellamy, Am. J. Vet. Res., in press). ETEC strain B44 previously cultured on Trypticase soy agar slants was subcultured every 24 h in Trypticase soy broth. When needed for challenge, broth cultures were seeded onto the surface of Minca agar containing 1% IsoVitaleX (10) (BBL) and grown at 37°C for 10 h. The bacterial growth was washed off the surface of the agar, suspended in PBS, and diluted to the desired concentration. The inoculum was administered into the back of the mouth from a 20-ml syringe when the calves were 12 to 14 h old. Initially, all calves were inoculated with 60 ml of PBS suspension containing 6×10^{11} viable B44. Later, when it became apparent that the MSB was not protecting calves challenged with that dose, nine calves were challenged with 30 ml of suspension containing 3×10^{11} viable B44.

Collection of samples and clinical observations. Lacteal secretions were collected from each cow at parturition before the calf had nursed. Whey was prepared by using commercial renin and stored at −20°C until used for measurement of antibody titers.

Calves were examined at challenge, 6, 12, and 24 h postchallenge (PC), and then daily until 10 days of age. At each examination, fecal consistency and the degree of dehydration and of depression were recorded and used to determine a clinical score which ranged in value from 0 to 3 as follows: 0 = no diarrhea, dehydration, or depression; 1 = transient softness of feces or watery diarrhea which resulted in a loss of ≤4% of body weight at challenge (BW); 2 = diarrhea accompanied by a loss of 4 to 11% BW and depression; 3 = diarrhea accompanied by a loss of >11% BW and death. Calves were weighed at challenge and then 12 h and 1, 2, 3, 5, and 10 days PC.

Serology. Whey prepared from colostrum collected at parturition was examined for antibodies to K99, K30, and O9 antigens. Antibody titers against K99 were determined by a solid-phase radioimmunoassay which will be reported in detail elsewhere. Briefly, rabbit anti-bovine immunoglobulin G (RABIgG) was prepared and labeled with ^{125}I by the chloramine-T method of Greenwood et al. (9) as described previously (1). Microtiter plates (no. 3040, Falcon Plastics, Oxnard, Calif.) were coated with a solution containing 20 μg of K99 in PBS (50 μl per well) for 24 h at 4°C. The wells were washed four times with PBS containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20, Sigma Chemical Co., St. Louis, Mo.), and then incubated with 50 μl of a 1% solution of horse serum albumin. After 12 h, the wells were again washed four times with PBS–Tween, and 50 μl of various dilutions of test whey was added. Control wells received 50 μl of fetal calf serum (GIBCO, Canada). Plates were then incubated for 60 min at 37°C, washed four times in PBS-Tween, and 50 μl of ^{125}I-labeled RABIgG in PBS was added to each well. After a further incubation for 60 min at 37°C, the wells were again washed four times with PBS-Tween and then allowed to air dry at room temperature. The bottoms of the wells were punched out and counted for 1 min in a gamma counter (model 1185T; Nuclear Chicago, Chicago, Ill.). The amount of specific binding in each whey sample was expressed as the binding ratio, which was calculated from the counts per minute in test whey per counts per minute in fetal calf serum. A binding ratio of 4.0 was considered positive, based on examination of 17 precolostral serum samples taken from calves delivered by cesarean section.

Antibody titers to K30 antigen were determined by agglutination on slides, using a suspension of strain B44 grown on blood agar plates as the antigen. Culturing strain B44 on blood agar enhances development of capsular carbohydrate which interferes with detectability of K99 and O9 antigens (R. E. Isaacson, H. W. Moon, and R. A. Schneider, Am. J. Vet. Res., in press). Hence, strain B44 grown on blood agar failed to agglutinate in K99 or O9 antiserum, but reacted strongly with monospecific K30 antiserum prepared in rabbits by the method of Edwards and Ewing (4).

Antibody titers to O9 antigen were also determined by agglutination on slides, using a suspension of au-

toclaved B44 grown on blood agar as the antigen. Autoclaving destroys the K antigens (4), so the suspension of B44 reacted strongly with O9, but not with K99 or K30 antiserum.

RESULTS

All calves were observed standing and nursing their dam within 3 h of birth.

Clinical response of calves after challenge. In the control group, three of four calves challenged with 3×10^{11} cells of *E. coli* strain B44 developed severe diarrhea and died, whereas all six calves challenged with 6×10^{11} organisms died (Tables 1 and 2). All calves except no. 10-52 became diarrheic within 6 to 12 h, rapidly dehydrated, and died within 24 to 72 h PC. By 24 h PC, calves in this group lost an average of 14.2% BW (Table 2).

Calves born to cows immunized with purified K99 were protected against severe diarrhea and death after challenge with either dose of *E. coli*. Transient or mild diarrhea occurred in seven calves but only two (no. 5-26 and 9-50) lost more than 4% BW. Calves in this group gained an average of 0.2% BW by 24 h PC.

Calves born to cows immunized with the HWCB were also protected against severe diarrhea and death. Only three of nine calves became diarrheic and of these only one, no. 7-33, died.

In contrast to calves in the two vaccinated groups described above, calves born to cows immunized with the MSB were not protected against fatal diarrhea. Four of the six calves in this group, including calf no. 7-51 which received lower challenge, died within 24 to 72 h PC. The age at the onset of diarrhea and the pattern of weight loss in these calves was very similar to those in the control group. The other two calves in this group, one of which received the lower challenge dose, also developed severe, but nonfatal, diarrhea which lasted 1 to 2 days. By 24 h PC, calves in this group lost an average of 11.2% BW. Calf no. 6-35 was born dead 34 days after the second vaccination. The cause of death was not determined.

Serological response. None of the cows in the control group had colostral antibody titers against K99 antigen of greater than 10 as measured by radioimmunoassay, or against K30 antigen, as measured by slide agglutination (Table 2). Five of the 10 cows in this group had low agglutinating antibody titers against O9. Cows vaccinated with purified K99 had colostral K99 antibody titers which ranged from 4,500 to 13,500, with a geometric mean titer (GMT) of 7,863 (Table 2). None of the cows in this group had titers against K30, whereas four of nine had low titers against O9.

TABLE 1. *Occurrence of diarrhea and death in calves after challenge with ETEC strain B44*

Vaccine group	Calves[a]	
	Diarrheic[b]	Dead
Nonvaccinated controls	9/10	9/10
Purified K99	2/9	0/9
HWCB	2/9	1/9
MSB	6/6[c]	4/6

[a] Values indicate number of calves affected per number challenged.

[b] Clinical score of 2 or greater. See text for details of classification.

[c] Seventh calf in this group born dead: not challenged.

The K99 titers in cows in the HWCB group averaged approximately one-half of those in the purified K99 group but were still significantly higher than in the control cows. In addition, K30 and O9 antibody titers were increased in cows in this group as compared to those in the control group. The MSB either failed to stimulate, or stimulated only low levels of antibody against all three antigens.

There was a highly significant correlation between the clinical response of the calves after challenge and the colostral K99 antibody titer in their dams. Only 1 of 16 (6.3%) calves nursing dams with K99 titers greater than 1,000 died, whereas 12 of 15 (80.0%) calves nursing dams with K99 titers of 1,000 or less died of severe diarrhea after challenge ($x^2 = 14.40$; $P < 0.0005$) (Table 2). There was no statistical correlation between the clinical response of calves and either K30 or O9 colostral antibody titers.

DISCUSSION

Immunization of pregnant cows can be used to stimulate the development of lacteal immunity against experimentally induced and naturally occurring colibacillosis in calves. Myers demonstrated that colostral antibody against the A-type polysaccharide or capsular K30, but not the somatic O antigen of ETEC strain B44, prevented diarrhea in calves experimentally challenged with the homologous organism (18). No attempt to demonstrate cross-protection between different capsular K antigens of ETEC has been reported. Since a variety of different capsular K antigens occur on bovine ETEC (19, 24), a vaccine based on developing immunity to these antigens may have to contain capsular components from several different serotypes to provide protection against a wide variety of ETEC strains.

An alternative approach to that described above would be to develop immunity to a com-

TABLE 2. *Clinical score, change in BW, and colostral antibody titers in calves and cows in each vaccine group*

Vaccine	Cow-calf no.	Clinical score[a]	% Change in BW 24 h PC	Reciprocal of colostral antibody titer[b]		
				K99	K30	O9
Nonvaccinated controls	1-1	3[c]	−21.4	ND[d]	ND	ND
	2-13	3	−10.0	<10	−[e]	−
	3-14	3	−23.5	<10	−	UD[f]
	4-30	3	−12.2	<10	−	−
	5-31	3	Dead	10	−	−
	6-43	3[c]	−18.8	<10	−	2
	7-44	3	−16.0	<10	−	−
	8-45	3[c]	−11.7	10	−	4
	9-48	3	−13.9	<10	−	2
	10-52	1[c]	0.0	<10	−	16
			−14.2 ± 7.0[g]	<10[h]	−	1.9
Purified K99	1-5	0[c]	−1.5	4,500	−	UD
	2-10	1	+1.6	ND	ND	ND
	3-11	1	−1.2	5,800	−	−
	4-17	1	+4.6	6,000	−	4
	5-26	2	−6.8	7,600	−	−
	6-27	0	+3.0	10,400	−	−
	7-37	1	+1.2	11,500	−	−
	8-38	1	+5.8	13,500	−	UD
	9-50	2	−5.1	7,600	−	2
			+0.2 ± 4.2[g]	7,863[h]	−	1.3
HWCB	1-4	1[c]	0.0	6,000	4	2
	2-6	0	+3.7	1,300	4	128
	3-7	0[c]	+1.3	12,700	8	16
	4-15	0	+1.5	ND	ND	ND
	5-19	0	+2.7	3,200	8	32
	6-23	0	+1.6	3,300	4	4
	7-33	3	−13.6	2,100	8	8
	8-36	2	−1.4	2,300	16	64
	9-46	0	+1.3	6,000	8	32
			−0.3 + 5.2[g]	3,632[h]	6.7	17.4
MSB	1-18	3	−11.6	240	16	32
	2-20	2	−13.6	100	4	−
	3-21	3	−15.2	−	UD	−
	4-28	3	−11.1	50	2	4
	5-29	2[c]	−5.7	−	4	−
	6-35	NC[i]	NC	ND	ND	ND
	7-51	3[c]	−10.0	560	2	4
			−11.2 ± 3.3[g]	30[h]	3.2	2.8

[a] See text for details of classification.
[b] K99 antibody titers were determined by radioimmunoassay; K30 and O9 antibody titers were determined by slide agglutination.
[c] Challenge dose was 3×10^{11} cells; all other calves were challenged with 6×10^{11} cells of ETEC strain B44.
[d] ND, Not determined.
[e] −, Negative.
[f] UD, Undiluted.
[g] Value represents mean ± standard deviation.
[h] Value represents geometric mean titer.
[i] NC, Not challenged: calf born dead.

mon antigen possessed by many different ETEC strains. The K99 antigen seemed to us to be a logical choice for investigation for the following reasons. First, it is present on the majority of bovine ETEC regardless of serotype (19, 24). Second, it plays a vital role in the pathogenesis of the disease by allowing the ETEC to adhere to the intestinal mucosa and proliferate to abnormally high numbers (12, 26). In addition, previous work demonstrated that lacteal immu-

nity against K99, K88, and 987 pili prevented severe diarrhea and death in piglets experimentally challenged with porcine ETEC possessing the homologous colonization factor (17, 12a, 22, 25). Third, the antigen can be purified by using current technology (11).

The data presented here indicate that lacteal immunity against the K99 antigen prevented severe diarrhea and death in calves experimentally challenged with ETEC strain B44. Twelve of 15 (80.0%) calves nursing cows with colostral K99 antibody titers ≤1,000 developed severe diarrhea and died after challenge, whereas only 1 of 16 (6.3%) calves nursing cows with titers >1,000 died ($P < 0.005$). In addition, the colostrum of nonvaccinated control cows did not contain antibody to K99, and 9 of 10 (90%) calves succumbed to fatal diarrhea within 72 h PC. On the other hand, the colostrum of cows vaccinated with purified K99 contained high levels of K99 antibody, no K30 antibody, and low levels of O9 antibody which did not differ from levels seen in control cows. In this group, all of nine (100%) calves were protected against fatal diarrhea.

In contrast to the above, there was no statistical correlation between protection against fatal diarrhea and the levels of either K30 or O9 colostral antibody which occurred in this study. However, the contribution of these antibodies to protection in the HWCB group cannot be eliminated from consideration because, in most cases, colostrum which contained increased antibody titers to these two antigens also contained increased titers to K99. Six of nine calves in the HWCB group, as compared to only two of nine calves in the K99 group, did not develop even a mild diarrhea after challenge. Since the GMT of K99 antibody in the HWCB group was less than half that in the K99 group, it is likely that K30 or O9 antibody, in addition to K99 antibody, contributed to protection. Other studies showed that K30 but not O9 antibody prevented diarrhea caused by strain B44 (18). Since the K30 titers observed in this study were quite low, it is possible that higher titers could be protective even in the absence of K99 antibody.

The findings reported here are consistent with the hypothesis that K99 antigen is a necessary virulence attribute of bovine ETEC and that lacteal immunity against this pilus prevents diarrhea, probably by blocking adhesion of the bacteria to the intestinal mucosa. Purified or semi-purified K99, K88, and 987 pili as well as bacterins containing pilus-positive whole cells have now been shown to be safe and effective vaccines against enterotoxigenic colibacillosis in suckling calves and piglets (12a, 17-20, 22, 23, 25). Additional studies are required to determine which type of preparation will be most useful.

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